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Morphine modulates cell proliferation through *mir133b* & *mir128* in the neuroblastoma SH-SY5Y cell line ☆

Veronica Gonzalez-Nunez [a,b,c], Jose Antonio Noriega-Prieto [b,c], Raquel E. Rodríguez [a,b,c,*]

[a] Department of Biochemistry and Molecular Biology, Faculty of Medicine, University of Salamanca, Spain
[b] Instituto de Neurociencias de Castilla y León (INCyL), University of Salamanca, Spain
[c] Instituto de Investigación Biomédica de Salamanca (IBSAL), Spain

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ABSTRACT

Neuroblastoma is a childhood cancer with high incidence and high mortality rate. Great efforts are made to find new treatments and molecular markers for diagnosis and prognosis. miRNAs stand for novel strategies to modulate tumor growth, as they can act either as tumor suppressors or as oncogenes. Morphine is an opioid agonist widely used to treat severe and chronic pain, as for example cancer pain. Previous studies have revealed that morphine is able to modify cancer progression, by acting on proliferation or on apoptosis; however, up to date, the available results are contradictory, maybe due to the different doses used, routes of administration and model systems. While some studies show that morphine promotes cell proliferation and metastasis, other authors sustain that morphine effect is mainly antiproliferative and pro-apoptotic. In this study we aim to establish the effect of chronic opiate administration on cell proliferation in the neuroblastoma SH-SY5Y cell line. Low doses of morphine (10 nM) promoted cell proliferation in undifferentiated cells and reduced the expression levels of *miR133b*, while higher doses (1 μM) inhibited cell proliferation and correlated with decreased levels of *miR133b* and *miR128* without triggering apoptosis. Naloxone, the classical opioid antagonist, could not fully block the effect of morphine on *miR128* expression, so that the observed effect may be mediated by non-opioid mechanisms. Our results represent a further contribution to the hypothesis that a joint regulation of miRNA networks and the specific characteristics of the target tissue may determine the effect of morphine on tumor cell growth.

1. Introduction

Neuroblastoma is a tumor that appears in childhood with high incidence and mortality rate. This tumor of the nervous system occurs during the embryonic stage or at the early postnatal age, and impairs the growth of sympathetic cells derived from the neural crest. It is the most common malignant tumor in infants, besides being responsible for a dramatic percentage of pediatric cancer [1]. Thus, great efforts are made to find new treatment strategies and molecular markers for diagnosis and prognosis. miRNA targeting stands for a novel approach to modulate tumor growth, as it can be the case of neuroblastoma [1,2].

miRNAs are small non-coding single-stranded RNAs of approximately 22 bp, that act as regulators of gene expression at the post-transcriptional level. It has been estimated that about 30% of all human genes are regulated by miRNAs by binding to their 3′UTR [3]. It is known that miRNAs are involved in the control of neural development by modulating cell proliferation, differentiation, adult neurogenesis and apoptosis [4,5]. Besides, miRNAs can control some neuronal functions like plasticity [5] and miRNA homeostasis is linked to some pathological states [6], as for example neurological disorders like Parkinson disease [5,7]. miRNAs are also deregulated in many tumors, and they can act either as tumor suppressors or oncogenes, depending on their targets [5]. Therefore miRNAs and other non-coding RNAs arise as novel therapeutic strategies in cancer research [8]. In fact, it has been already proven that mice with neuroblastoma show differences in pro-apoptotic and anti-angiogenic activities when treated with nanoparticles encapsulating miR-34a, which specifically targets this type of tumor [9].

miR133b is specifically expressed in a subpopulation of dopaminergic cells located in the midbrain, and regulates their differentiation, maturation and neuronal function [7,10]. This miRNA is also deregulated in several types of cancer, as for example colorectal [11,12] and bladder cancer [13]. It has also been reported that miR133b targets two prosurvival molecules which are members of the *BCL-2* (B-cell CLL/lymphoma 21 2) family in adenocarcinoma cell lines [14].

Abbreviations: BCL-2, B-cell CLL/lymphoma 21 2; BDNF, brain derived neurotrophic factor; DAPI, 4′,6-diamidino-2-phenylindole dihydrochloride; dcx, doublecortin; ERK, extracellular signal-regulated kinase; M, morphine; Nx, naloxone; OGFR, opioid growth factor receptor; *OPRM1*, μ opioid receptor; RA, retinoic acid; RTK, receptor tyrosine kinase; TRK3, neurotrophin-3 receptor gene; UTR, untranslated region

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* Corresponding author at: Dept. Biochemistry and Molecular Biology, Faculty of Medicine, Instituto de Neurociencias de Castilla y León (INCyL), c/ Pintor Fernando Gallego n. 1, 37007 Salamanca, Spain. Tel.: +34 923 294400x4626/5301.
E-mail address: requelmi@usal.es (R.E. Rodríguez).

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The brain-enriched *miR128* is transcribed from two genes (miRNA128-1 and miR128-2) which give rise to two identical miRNAs in their mature form [15]. Studies performed in the neuroblastoma SH-SY5Y cell line indicate that *miR128* represses the expression of the truncated isoform of the human neurotrophin-3 receptor gene (NTRK3), and modulates the expression of genes involved in cytoskeletal reorganization, apoptosis, cell survival and proliferation. Overexpression of *miR128* up-regulates the anti-apoptotic gene BCL-2 [16]. Another study performed in the same cell line revealed that *miR128* targets and down-regulates the expression of reelin and doublecortin (DCX), two genes which are related to neuroblastic migration; therefore *miR128* decreases neuroblastoma growth and cell motility, thus affecting invasiveness [15].

Morphine is an opioid agonist widely used to treat severe and chronic pain, as for example cancer pain. It has been shown that, apart from its analgesic properties, morphine elicits other side effects. Previous studies have revealed that morphine is able to modify cancer progression, either by acting on proliferation or on apoptosis [17]. However, up to date, the published results are contradictory [18], and some authors raise the question whether the μ opioid receptor might not play a significant role in the regulation opioid-mediated tumor growth [17]. Several studies indicate that the opioid growth factor receptor (OGFR) can be involved in the modulation of tumor progression elicited by morphine [19]. *In vivo* experiments revealed that the duration of the opioid receptor blockade by naltrexone modulates neuroblastoma progression [20]. Some other studies pointed out that morphine can promote tumor growth due to its inhibitory effect on the immune response [21]. Other authors have shown that that the μ opioid receptor expression is increased in several non-small cell lung cancer cell lines as well as in lung tissue from patients with this type of carcinoma, and that μ opioid receptor activation could be correlated with cell growth and metastasis [22].

SH-SY5Y is a human-derived cell line that was developed from the SK-N-SH cell line, which was obtained from a neuroblastoma. Interestingly, the SH-SY5Y cell line endogenously expresses the μ opioid receptor, which is the pharmacological target for morphine [23]. Exposure to retinoic acid and/or BDNF (Brain Derived Neurotrophic Factor) can trigger differentiation of SH-SY5Y cells into a homogeneous population of cells which expresses neuronal markers and shows a neuron-like phenotype [24]. Previous results from our group revealed that morphine might be regulating cell proliferation in immature neurons by controlling the expression of miR133b in zebrafish [10]. Given the importance of morphine in the medical practice, and especially in the management of cancer pain, in this study we aimed to establish the effect of different doses of morphine on cell proliferation in the neuroblastoma SH-SY5Y cell line. Besides, we want to determine if the proliferative effect is due to the interaction of morphine with the μ opioid receptor and whether this effect is mediated by miRNAs, namely *miR133b* and *miR128*.

2. Materials and methods

2.1. Drugs and chemicals

Morphine hydrochloride was provided by the Spanish Ministry of Health and naloxone (Nx) was purchased from Sigma-Aldrich. All other reagents used were from analytical grade.

2.2. Cell culture and drug treatments

The neuroblastoma SH-SY5Y cell line (a kind gift of Dr. D. Martinez-Zanca) was cultured in DMEM (Dulbecco's modified Eagle's medium): HAM F-12 (1:1) supplemented with 10% (v/v) fetal calf serum, 2 mM glutamine, 100 U mL^{-1} penicillin and 0.1 mg mL^{-1} streptomycin (all from Gibco-BRL, Life Technologies), at 37 °C in humidified atmosphere containing 5% (v/v) CO_2 in a Forma incubator. Cells were harvested with 2 mM EDTA in PBS and plated 1 day before the experiment, so that they reached 70% of confluence on the next day. Cells were divided into five experimental groups: 1) control group (fresh medium without drug), 2) 10 nM morphine, 3) 1 μM morphine, 4) 10 μM naloxone and 5) 10 μM naloxone + 1 μM morphine (cells were preincubated with 10 μM naloxone for 1 h prior to the treatment with 1 μM morphine). Cells were further cultured for 24 h and then processed as needed.

2.3. Retinoic acid-induced differentiation of SH-SY5Y neuroblastoma cell line

To induce the cellular differentiation by retinoic acid in vitro, we have followed the procedure described in [24]. Briefly, cells were seeded at 65% confluency 1 day in advance and cultured in DMEM with 15% (v/v) fetal calf serum. Cells were exposed to 10 μM retinoic acid in 0.1% ethanol for 8 days and medium was changed every other day. After 8 days, cells were detached from the flask and exposed to different drug treatments as established before.

2.4. Immunocytochemical studies

Cells were grown on poly-lysinated coverslips which were put on 12-well plates (2 coverslips per well) and exposed to drug treatments as previously described. After 24 h incubation, cells were fixed with 4% paraformaldehyde and washed with PBST. For proliferation assays, the mouse anti-histone 3 (phospho Ser^{10}) (Abcam) was used as primary antibody and Alexa Fluor 488 conjugated anti-mouse (Molecular Probes, Life Techonologies) as secondary antibody. Negative controls (no primary or secondary antibody) were performed in parallel. TUNEL assays to determine apoptosis were performed using the In Situ Cell Death Detection Kit, AP (Roche Applied Science) following the manufacturer's instructions. Positive (cells treated with DNase I) and negative (no TDT enzyme) controls were performed in parallel. Cell nuclei were counterstained with 4,6-diamidino-2-phenylindole (DAPI) (Roche Applied Science). Experiments were performed in duplicates and repeated three times.

Samples were analyzed with an Olympus AX70 fluorescence microscope. Five images were taken from each treatment and for each experimental series. Image analysis and cell counting were performed with ImageJ software (NIH). The percentage of cells in proliferation or in apoptosis was normalized to the total number of cells and data were represented and analyzed by using GraphPad Prism software (San Diego, CA, USA).

2.5. Quantitative real-time PCR analysis

Cells were exposed to the different drug treatments, then harvested and the cell pellet was stored at −80 °C until use. Total RNA from cells was isolated by using the Trizol reagent (Invitrogen, Life Technologies) and treated with DNase I (Roche Applied science). For *GAPDH* and *OPRM1* genes, 1 μg RNA was reversed transcribed by using the M-MuLV retrotranscriptase (New England Biolabs) and random hexamers. To perform the real-time PCR on miRNAs (both *miR133b* and *miR128*), cDNA was synthesized following the procedure described in [25]. Briefly, prior to the cDNA synthesis, total RNA was polyadenylated with poly-A polymerase (New England Biolabs) and then reverse transcribed with the M-MuLV retrotranscriptase (New England Biolabs). The sequence of the qPCR primers is given in Table 1. The amplification reactions were carried out in a final volume of 25 μL by using Power SYBR Green PCR Master Mix (Applied Biosystems, Life Techonologies) and the real-time polymerase chain reaction was performed by using the ABI Prism 7300 (Applied Biosystems). Experiments were conducted in triplicates and repeated four times. Ct values were calculated with the SDS v1.3.1 software (Applied Biosystems, Life Technologies) for each gene and the cDNA abundance of each transcript was calculated relative to the expression of the housekeeping gene *GAPDH* using the REST-384© v2 software [26]. Also, standard curves for each gene and negative controls were included in each PCR reaction. Statistical analysis was performed by using GraphPad Prism software (San Diego, CA, USA).

Table 1
Primers used for qPCR analysis. Summary of the primers that have been used in qPCR experiments, ENSEMBL gene ID and amplicon length.

Gene	ENSEMBL gene ID	qPCR primer sequence	Amplicon length
miR133b	ENSG00000199080	F: 5′ GTTTGGTCCCCTTCAAC 3′ R: 5′ AGTTTTTTTTTTTTTTTAGCTG GTT 3′	39 nt
miR128	ENSG00000207654	F: 5′ CACAGTGAACCGGTCT 3′ R: 5′ GTCCAGTTTTTTTTTTTTTTTAAA GAG 3′	41 nt
oprm1	ENSG00000112038	F: 5′ CGGAAACTTCCTGGTCATGT 3′ R: 5′ CTGCCAGAGCAAGGTTGAA 3′	89 nt
gapdh	ENSG00000111640	F: 5′ CTGACTTCAACAGCGACACC 3′ R: 5′ CCCTGTTGCTGTAGCCAAAT 3′	120 nt

3. Results

3.1. Changes in proliferation in the SH-SY5Y neuroblastoma cell line elicited by morphine

To determine if morphine can alter proliferation in a neuroblastoma model, the SH-SY5Y cell line was incubated with different doses of morphine, 10 nM (a subeffective dose) and 1 μM (an analgesic dose). To establish if the proliferative effect is mediated by the m opioid receptor, the universal antagonist naloxone was also used at 10 mM, alone and in combination with 1 mM morphine. An antibody against the mitotic marker phospho-histone 3 (Ser^{10}) was used to label the proliferative cells, images were acquired, mitotic and total nuclei were counted and the percentage of cells in proliferation was calculated. As seen in Figs. 1 and 4a, there was a statistically significant increase of phospho-histone 3 positive nuclei in the group of cells incubated with the lower dose of morphine (10 nM), and a decrease in those cells exposed to the higher dose (1 μM). No changes were seen when the cells were incubated with naloxone, either alone or in co-incubation with morphine.

In order to assess if morphine could also be affecting apoptosis and thus explaining the changes in cell proliferation, TUNEL assays were performed (Fig. 2). Quantification analysis revealed that there were no statistically significant changes in the percentage of apoptotic nuclei for any of the tested doses of morphine and naloxone (Fig. 4b).

3.2. Morphine does not alter proliferation in differentiated SH-SY5Y cells

The SH-SY5Y cell line was cultured with retinoic acid to promote cell differentiation into a neuronal phenotype; then cells were treated with morphine and naloxone as previously described. Proliferation studies were conducted and quantification of cell proliferation was performed

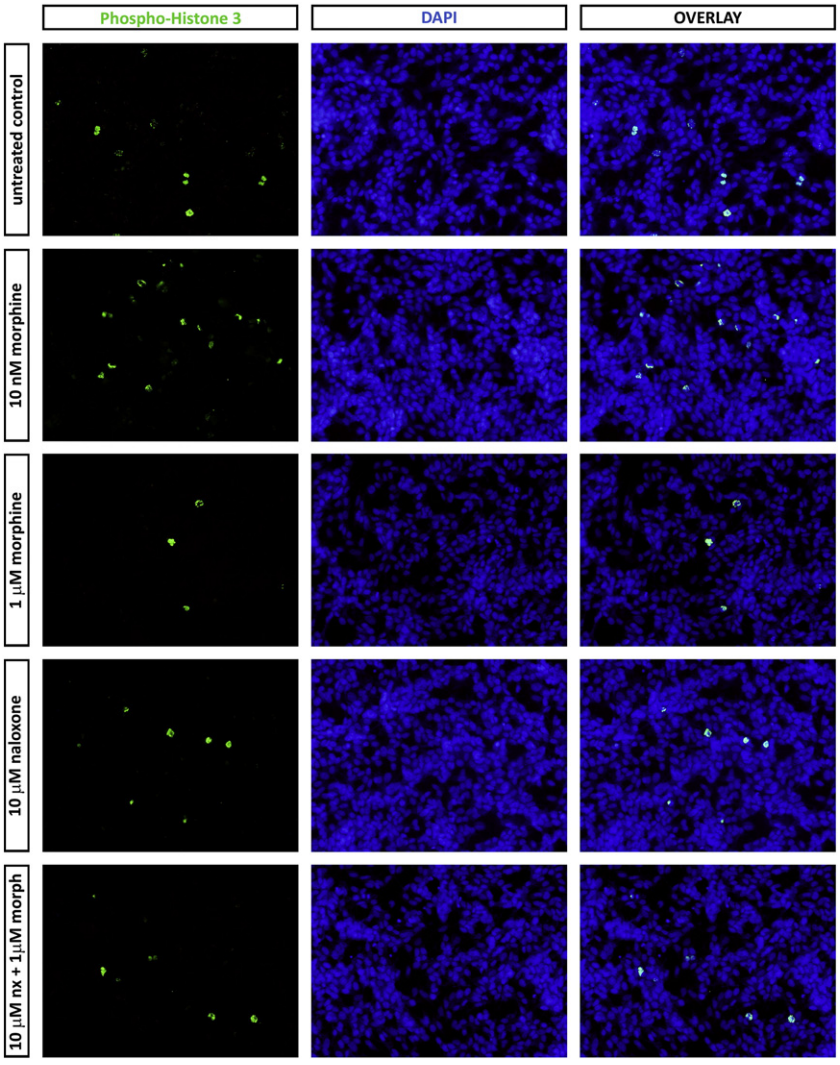

Fig. 1. *Morphine disrupts proliferation in the neuroblastoma SH-SY5Y cell line.* Cells were treated with different concentrations of morphine and/or naloxone, and proliferation was assayed. Phospho-histone 3 (Ser^{10}) immunostaining (green) has been used to label mitotic cells and the cell nuclei have been counterstained with DAPI (blue). Note that 10 nM morphine induced an increase in cell proliferation, whereas the 1 μM dose reduced the number of phospho-histone 3 positive nuclei, as compared to untreated cells, and cells incubated with 10 μM naloxone (alone or in co-incubation with morphine).

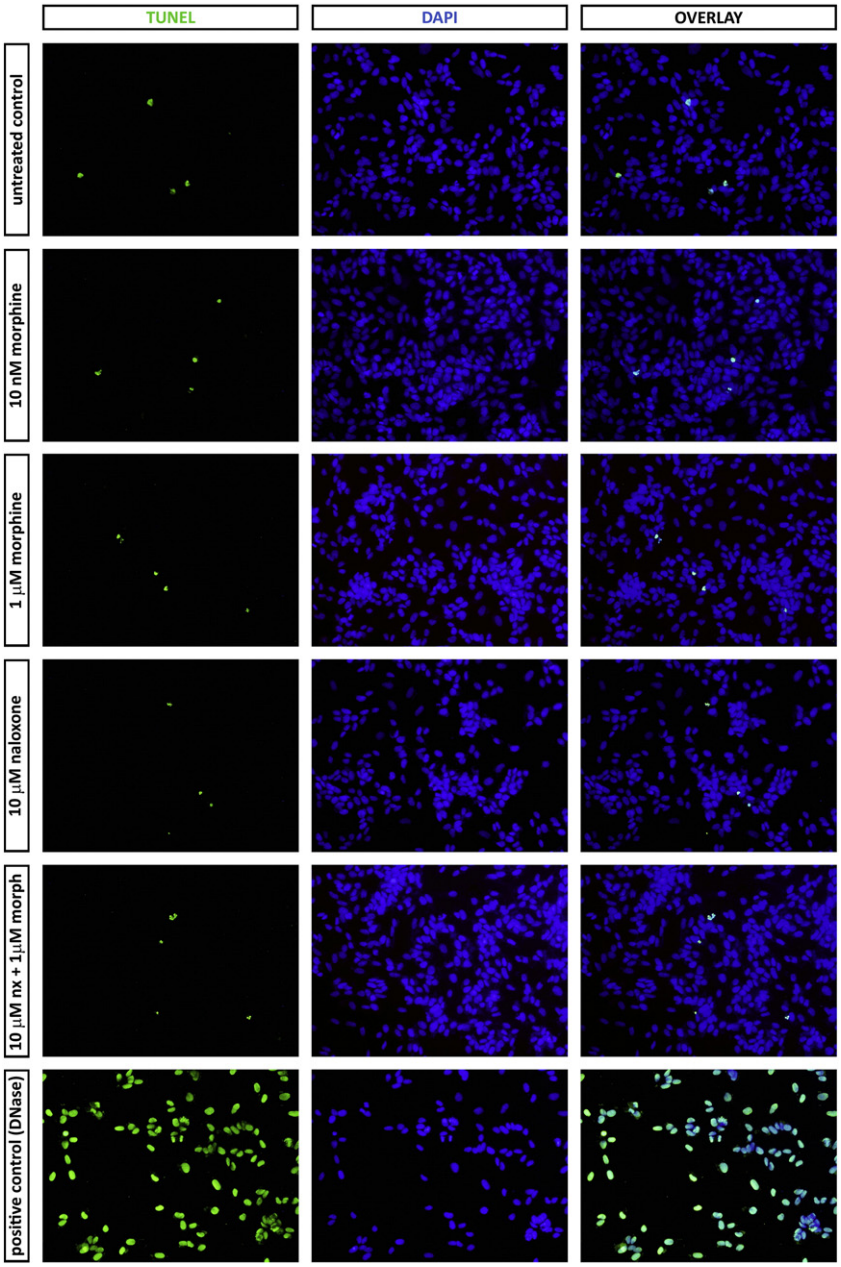

Fig. 2. *Apoptosis assays on the neuroblastoma SH-SY5Y cell line after opiate treatment.* To determine if opiate treatment promotes apoptosis, cells were exposed to different concentrations of morphine and/or naloxone, and programmed cell death was determined by TUNEL staining. Apoptotic nuclei were labeled in green and total nuclei were counterstained with DAPI (blue).

(Figs. 3 and 4c). In this case, none of the two morphine doses was able to alter the fraction of nuclei labeled with phospho-histone 3 antibody after retinoic acid treatment; naloxone had no affect on cell proliferation, neither alone nor in co-incubation with morphine.

3.3. miR133b and miR128 are differentially regulated by morphine in SH-SY5Y cells

We next questioned if treatment with different doses of morphine could alter the expression levels of *miR133b* and *miR128* in the SH-SY5Y cell line. Real-time PCR analysis allowed us to quantify the expression levels of these two miRNAs, and results are summarized in Table 2. *miR133b* followed a dose–response curve, as 10 nM morphine reduced about 30% of *miR133b* expression, but its levels dropped down up to 65% when the 1 μM dose was used. No statistically significant changes were observed when cells were incubated with naloxone, either alone or in co-incubation with morphine. In the case of *miR128*, the effect of morphine showed a biphasic model, as the 10 nM dose increased *miR128* levels (20% increase, but not statistically significant), but they were significantly reduced when cells were incubated with 1 μM morphine (65% reduction). No changes in *miR128* expression were seen after 10 μM naloxone treatment. Interestingly this opioid antagonist was not able to fully reverse the effect of morphine, as the co-incubation with 10 μM naloxone and 1 μM morphine reduced the expression levels of *miR128* about 38%. On a further step, we wanted to rule out that the differential effect of morphine could be due to changes in the expression of the μ opioid receptor after drug treatment. To prove this, real-time PCR assays were performed, and no significant changes in the expression levels of *OPRM1* (the human opioid receptor mu gene) were seen after any of the drug treatments used in this study.

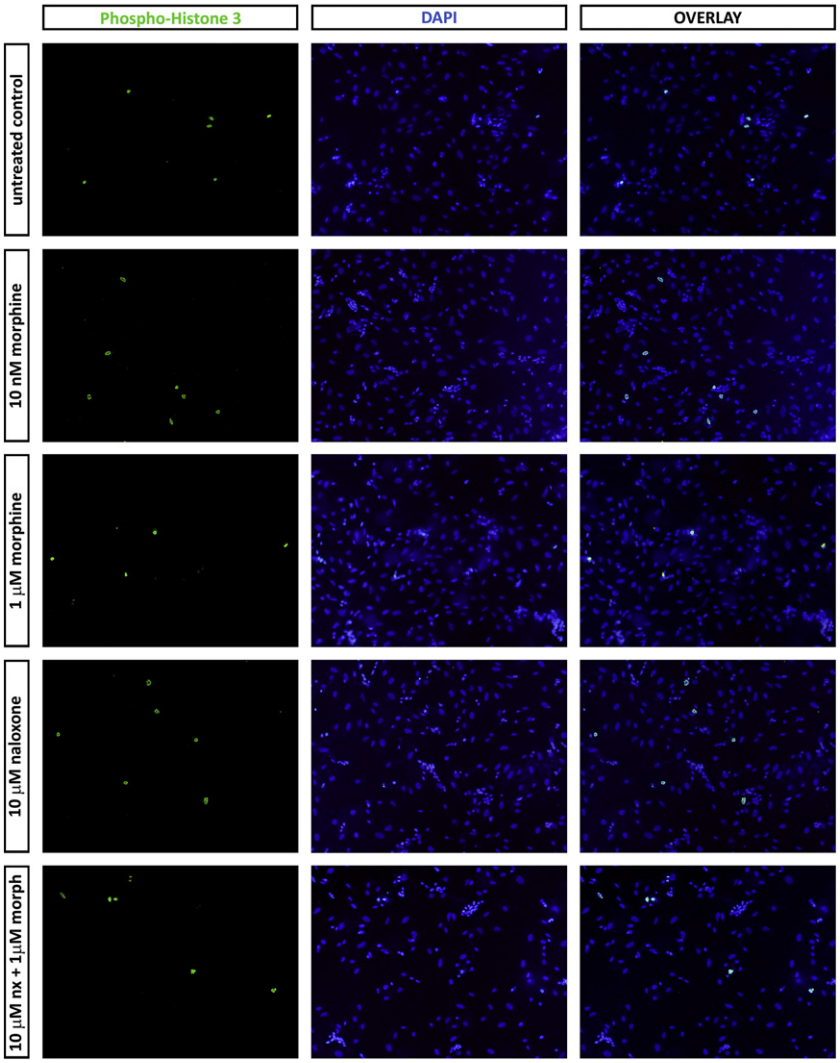

Fig. 3. *Proliferation of retinoic acid-differentiated SH-SY5Y cells after opiate treatment.* Cells were cultured with retinoic acid to promote differentiation into neuron phenotype and then were treated with 10 nM, 1 μM morphine, 10 μM naloxone and 1 μM morphine + 10 μM naloxone. Phospho-histone 3 (Ser^{10}) immunostaining (green) labels mitotic cells and the cell nuclei have been counterstained with DAPI (blue).

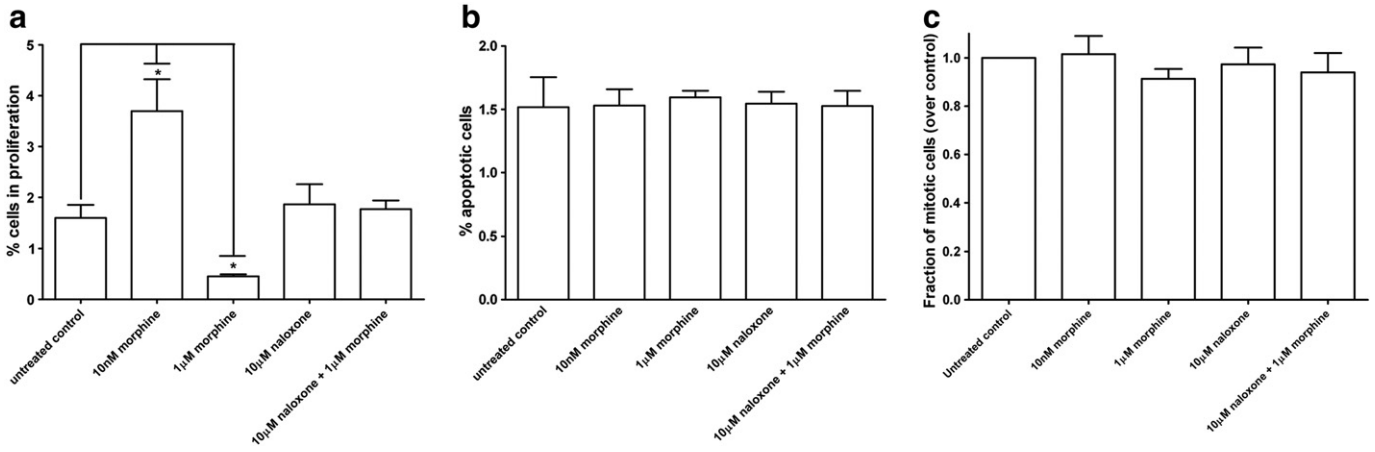

Fig. 4. *Quantification analysis of a) SH-SY5Y cells in proliferation and b) apoptotic cells after opioid treatment and c) retinoic acid-differentiated SH-SY5Y cells.* Data represent the mean ± S.E.M. of the percentage of undifferentiated SH-SY5Y cells in *a*) proliferation (phospho-histone 3 positive) and *b*) in apoptosis (TUNEL positive) of the total number of cells (cell nuclei counterstained with DAPI) in untreated controls and after treatment with different doses of morphine and naloxone. c) Fraction of retinoic acid-differentiated SH-SY5Y cells undergoing mitosis after opiate treatment. Data obtained from the drug treatments were compared to data from untreated controls and results were analyzed by unpaired *t* test with Welch's correction using Graph Pad Prism software. * — $p < 0.05$; ** — $p < 0.01$; *** — $p < 0.001$.

Table 2
Quantitative gene expression analysis of miR133b, miR128 and OPRM1 measured by real-time PCR in the neuroblastoma SH-SY5Y cell line after opiate treatments. Results represent the relative gene expression fold-changes for *miR133b*, *miR128* and *OPRM1* in SH-SY5Y cells that were exposed to different doses of morphine and/or naloxone. Data shown are normalized to *GAPDH*. Results were analyzed by one-way ANOVA followed by Tukey's post-hoc test; asterisks indicate statistical significance (* — $p < 0.05$ and *** — $p < 0.001$).

	Morphine 10 nM	Morphine 1 μM	Naloxone 10 μM	Naloxone 10 μM + morphine 1 μM
miR133b	0.70 ± 0.07 (*)	0.36 ± 0.07 (***)	0.76 ± 0.09	0.95 ± 0.06
miR128	1.21 ± 0.08	0.37 ± 0.04 (***)	0.96 ± 0.09	0.62 ± 0.05 (***)
OPRM1	1.12 ± 0.19	1.34 ± 0.27	1.33 ± 0.26	1.30 ± 0.30

4. Discussion

It is well known that morphine administration can alter tumor growth; however the evidence obtained up to now is rather conflicting, maybe due to the different doses used, routes of administration and model systems (i.e.*in vivo* or *in vitro* models). Even more, it has been proposed that some of the proliferative effects triggered by morphine could be caused by non-opioid mechanisms [18]. In this study we have used a cell line derived from a neuroblastoma and which endogenously expresses the μ opioid receptor. We have analyzed the effect of two different doses of morphine, 10 nM (a subpharmacological dose) and 1 μM (an analgesic dose) and whether the outcome can be blocked by the universal opiate antagonist naloxone when used at higher concentrations than morphine.

Our results indicate that low doses of morphine (10 nM) promote cell proliferation and reduce the expression levels of *miR133b* (Table 3). On its turn, analgesic doses of morphine (1 μM) inhibit proliferation, which is significantly correlated with decreased levels of both miRNAs, *miR133b* and *miR128*. These effects were only seen in undifferentiated cells, as no changes in the proliferation rate were found for retinoic acid-differentiated SH-SY5Y. None of the treatments altered the expression levels of *OPRM1*, so that changes in the transcriptional activity of the μ opioid receptor gene cannot be responsible for the proliferative effects of opiate agents. Besides, none of the drug treatments used in this study caused any changes in the apoptosis rate, thus ruling out the possibility that 1 μM morphine was triggering programmed cell death rather that blocking proliferation.

Interestingly, coincubation of morphine and naloxone (the classical opioid antagonist) causes a decrease in *miR128* levels. Therefore, while the effect of morphine on proliferation and on the expression of *miR133b* is blocked by naloxone, this opioid antagonist is not able to fully reverse the effect of morphine on *miR128* expression. It is hence probable that this effect is not due to a classical opioid interaction that can be blocked by the universal antagonist. In this line of thinking, it has been previously shown that non-opioid actions of opioid peptides can be elicited via the opioid growth factor receptor (OGFR), which has antiproliferative effects [27]. In fact, the OGFR is expressed in several types of cancer, such as pancreatic and thyroid cancer [28,29], and overexpression of OGFR promotes inhibition of cell growth via a μ-receptor independent mechanism.

It is well known that the opioid receptors are able to activate the MAPK signaling pathway after agonist binding; this intracellular cascade controls cell proliferation, differentiation and apoptosis [30]. It has been shown that μ and δ agonists stimulate the proliferation of neural progenitors in the adult hippocampus via ERK1/2 [31]. Our proliferation results are in accordance with previous studies, which showed that lower doses of morphine promoted tumor growth, but higher doses were able to inhibit metastasis [22]. Besides, it is well known that miRNAs can be modulating proliferation and that they are deregulated in some cancers. Here we show that the opposing effects of morphine on tumor growth could be due to differential modulation of miRNAs, in our case *miR133b* and *miR128*. Decreased levels of *miR133b* have been correlated with poor survival and metastasis in patients with colorectal cancer [11] and prostate cancer [32]. *miR133b* targets pro-survival genes of the *BCL-2* family (*MCL-1* and *BCL2L2*), and its expression levels are reduced by 28-fold in lung cancer [14]. Ectopic overexpression of *miR133b* also inhibits cell growth in prostate cancer by targeting the epidermal growth factor receptor [33]. On its turn, *miR128* regulates cell survival and proliferation by targeting the truncated NTRK3 receptor in the SH-SY5Y cell line [16]. There is evidence that *miR128* can act as anti-oncogenic and pro-differentiation factor. *miR128* is repressed in human glioma tumors and the reduction in the expression levels correlates with malignancy: *miR128* targets mitogenic tyrosine kinases (RTKs), so a down-regulation of *miR128* will release RTKs from its inhibitory effect [34]. In human neuroblastomas, *miR128* expression has been related to better prognosis and less invasiveness properties [15].

5. Conclusion

Chronic morphine displays a biphasic effect on cell growth in undifferentiated cells of the neuroblastoma SH-SY5Y cell line: while low doses of morphine (10 nM) enhance the proliferation rate, higher doses (1 μM) reduce the number of mitotic cells. The results that we present here indicate that morphine may be modulating proliferation through a joint action on *miR133b* and *miR128* in the neuroblastoma SH-SY5Y cell line. The effects elicited by morphine cannot be considered as universal mechanism, but rather a specific response for each cell type and tumor process. Our work supports the idea that a joint regulation of miRNA networks (in our case, *miR133b* and *miR128*) and the specific characteristics of the target tissue may determine the effect of morphine on tumor growth.

Declarations of interest

All authors declare that this manuscript contains no conflict of interest that would prejudice its impartiality.

Table 3
Summary table of the effect of opioid agents in the neuroblastoma SH-SY5Y cell line. Results are represented as % fold change when the tested groups are compared to control group (=100%); asterisks indicate statistical significance (* — $p < 0.05$ and *** — $p < 0.001$).

	10 nM morphine	1 μM morphine	10 μM naloxone	10 μM naloxone + 1 μM morphine
Proliferation in undifferentiated SH-SY5Y	231% (*)	28.38% (*)	117%	111%
Apoptosis	101%	105%	102%	100.6%
Proliferation in RA-differentiated SH-SY5Y	101.5%	91%	97%	94%
miR133b levels	70% (*)	36% (***)	76%	95%
miR128 levels	121%	37% (***)	96%	62% (***)
oprm1 mRNA levels	112%	134%	133%	130%

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References

[1] A.M. Davidoff, Neuroblastoma, Semin. Pediatr. Surg. 21 (2012) 2–14.
[2] W.C. Cho, OncomiRs: the discovery and progress of microRNAs in cancers, Mol. Cancer 6 (2007) 60.
[3] B.P. Lewis, C.B. Burge, D.P. Bartel, Conserved seed pairing, often flanked by adenosines, indicates that thousands of human genes are microRNA targets, Cell 120 (2005) 15–20.
[4] K.F. Meza-Sosa, D. Valle-Garcia, G. Pedraza-Alva, L. Perez-Martinez, Role of microRNAs in central nervous system development and pathology, J. Neurosci. Res. 90 (2012) 1–12.
[5] S. Martino, I. di Girolamo, A. Orlacchio, A. Datti, MicroRNA implications across neurodevelopment and neuropathology, J. Biomed. Biotechnol. 2009 (2009) 654346.
[6] E. van Rooij, The art of microRNA research, Circ. Res. 108 (2011) 219–234.
[7] J. Kim, K. Inoue, J. Ishii, W.B. Vanti, S.V. Voronov, E. Murchison, G. Hannon, A. Abeliovich, A microRNA feedback circuit in midbrain dopamine neurons, Science 317 (2007) 1220–1224.
[8] M. Esteller, Non-coding RNAs in human disease, Nat. Rev. Genet. 12 (2011) 861–874.
[9] A. Tivnan, W.S. Orr, V. Gubala, R. Nooney, D.E. Williams, C. McDonagh, S. Prenter, H. Harvey, R. Domingo-Fernandez, I.M. Bray, O. Piskareva, C.Y. Ng, H.N. Lode, A.M. Davidoff, R.L. Stallings, Inhibition of neuroblastoma tumor growth by targeted delivery of microRNA-34a using anti-disialoganglioside GD2 coated nanoparticles, PLoS One 7 (2012) e38129.
[10] F.M. Sanchez-Simon, X.X. Zhang, H.H. Loh, P.Y. Law, R.E. Rodriguez, Morphine regulates dopaminergic neuron differentiation via miR-133b, Mol. Pharmacol. 78 (2010) 935–942.
[11] P. Akcakaya, S. Ekelund, I. Kolosenko, S. Caramuta, D.M. Ozata, H. Xie, U. Lindforss, H. Olivecrona, W.O. Lui, miR-185 and miR-133b deregulation is associated with overall survival and metastasis in colorectal cancer, Int. J. Oncol. 39 (2011) 311–318.
[12] G. Hu, D. Chen, X. Li, K. Yang, H. Wang, W. Wu, miR-133b regulates the MET proto-oncogene and inhibits the growth of colorectal cancer cells in vitro and in vivo, Cancer Biol. Ther. 10 (2010) 190–197.
[13] T. Ichimi, H. Enokida, Y. Okuno, R. Kunimoto, T. Chiyomaru, K. Kawamoto, K. Kawahara, K. Toki, K. Kawakami, K. Nishiyama, G. Tsujimoto, M. Nakagawa, N. Seki, Identification of novel microRNA targets based on microRNA signatures in bladder cancer, Int. J. Cancer 125 (2009) 345–352.
[14] M. Crawford, K. Batte, L. Yu, X. Wu, G.J. Nuovo, C.B. Marsh, G.A. Otterson, S.P. Nana-Sinkam, MicroRNA 133B targets pro-survival molecules MCL-1 and BCL2L2 in lung cancer, Biochem. Biophys. Res. Commun. 388 (2009) 483–489.
[15] C. Evangelisti, M.C. Florian, I. Massimi, C. Dominici, G. Giannini, S. Galardi, M.C. Bue, S. Massalini, H.P. McDowell, E. Messi, A. Gulino, M.G. Farace, S.A. Ciafre, MiR-128 up-regulation inhibits Reelin and DCX expression and reduces neuroblastoma cell motility and invasiveness, FASEB J. 23 (2009) 4276–4287.
[16] M. Guidi, M. Muinos-Gimeno, B. Kagerbauer, E. Marti, X. Estivill, Y. Espinosa-Parrilla, Overexpression of miR-128 specifically inhibits the truncated isoform of NTRK3 and upregulates BCL2 in SH-SY5Y neuroblastoma cells, BMC Mol. Biol. 11 (2010) 95.
[17] M. Rasmussen, W. Zhu, J. Tonnesen, P. Cadet, E. Tonnesen, G.B. Stefano, Effects of morphine on tumour growth, Neuro Endocrinol. Lett. 23 (2002) 193–198.
[18] K. Gach, A. Wyrebska, J. Fichna, A. Janecka, The role of morphine in regulation of cancer cell growth, Naunyn Schmiedebergs Arch. Pharmacol. 384 (2011) 221–230.
[19] M. Schäfer, S.A. Mousa, Opioid therapy and tumor progression, Adv. Palliat. Med. 8 (2009) 53–56.
[20] I.S. Zagon, P.J. McLaughlin, Duration of opiate receptor blockade determines tumorigenic response in mice with neuroblastoma: a role for endogenous opioid systems in cancer, Life Sci. 35 (1984) 409–416.
[21] M. Ishikawa, K. Tanno, A. Kamo, Y. Takayanagi, K. Sasaki, Enhancement of tumor growth by morphine and its possible mechanism in mice, Biol. Pharm. Bull. 16 (1993) 762–766.
[22] B. Mathew, F.E. Lennon, J. Siegler, T. Mirzapoiazova, N. Mambetsariev, S. Sammani, L.M. Gerhold, P.J. LaRiviere, C.T. Chen, J.G. Garcia, R. Salgia, J. Moss, P.A. Singleton, The novel role of the mu opioid receptor in lung cancer progression: a laboratory investigation, Anesth. Analg. 112 (2011) 558–567.
[23] K.A. Horner, J.E. Zadina, Internalization and down-regulation of mu opioid receptors by endomorphins and morphine in SH-SY5Y human neuroblastoma cells, Brain Res. 1028 (2004) 121–132.
[24] M. Encinas, M. Iglesias, Y. Liu, H. Wang, A. Muhaisen, V. Cena, C. Gallego, J.X. Comella, Sequential treatment of SH-SY5Y cells with retinoic acid and brain-derived neurotrophic factor gives rise to fully differentiated, neurotrophic factor-dependent, human neuron-like cells, J. Neurochem. 75 (2000) 991–1003.
[25] I. Balcells, S. Cirera, P.K. Busk, Specific and sensitive quantitative RT-PCR of miRNAs with DNA primers, BMC Biotechnol. 11 (2011) 70.
[26] M.W. Pfaffl, G.W. Horgan, L. Dempfle, Relative expression software tool (REST) for group-wise comparison and statistical analysis of relative expression results in real-time PCR, Nucleic Acids Res. 30 (2002) e36.
[27] I.S. Zagon, M.F. Verderame, P.J. McLaughlin, The biology of the opioid growth factor receptor (OGFr), Brain Res. Brain Res. Rev. 38 (2002) 351–376.
[28] I.S. Zagon, M.F. Verderame, J. Hankins, P.J. McLaughlin, Overexpression of the opioid growth factor receptor potentiates growth inhibition in human pancreatic cancer cells, Int. J. Oncol. 30 (2007) 775–783.
[29] D. Goldenberg, I.S. Zagon, F. Fedok, H.S. Crist, P.J. McLaughlin, Expression of opioid growth factor (OGF)-OGF receptor (OGFr) axis in human nonmedullary thyroid cancer, Thyroid 18 (2008) 1165–1170.
[30] W. Zhang, H.T. Liu, MAPK signal pathways in the regulation of cell proliferation in mammalian cells, Cell Res. 12 (2002) 9–18.
[31] A.I. Persson, T. Thorlin, C. Bull, P.S. Eriksson, Opioid-induced proliferation through the MAPK pathway in cultures of adult hippocampal progenitors, Mol. Cell. Neurosci. 23 (2003) 360–372.
[32] J.P. Patron, A. Fendler, M. Bild, U. Jung, H. Muller, M.O. Arntzen, C. Piso, C. Stephan, B. Thiede, H.J. Mollenkopf, K. Jung, S.H. Kaufmann, J. Schreiber, MiR-133b targets antiapoptotic genes and enhances death receptor-induced apoptosis, PLoS One 7 (2012) e35345.
[33] J. Tao, D. Wu, B. Xu, W. Qian, P. Li, Q. Lu, C. Yin, W. Zhang, microRNA-133 inhibits cell proliferation, migration and invasion in prostate cancer cells by targeting the epidermal growth factor receptor, Oncol. Rep. 27 (2012) 1967–1975.
[34] T. Papagiannakopoulos, D. Friedmann-Morvinski, P. Neveu, J.C. Dugas, R.M. Gill, E. Huillard, C. Liu, H. Zong, D.H. Rowitch, B.A. Barres, I.M. Verma, K.S. Kosik, Pro-neural miR-128 is a glioma tumor suppressor that targets mitogenic kinases, Oncogene 31 (2012) 1884–1895.